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14. ABSTRACT

Fragile X syndrome (FXS), the most common heritable form of intellectual disability and most common single-gene form of autism, is caused by partial or complete silencing of the fragile X (*FMR1*) gene upon expansion of a CGG-repeat element within the gene, leading to loss of the *FMR1* protein (FMRP). The protein is important for brain development, and its loss is accompanied by both intellectual and behavioral disability. The purpose of the research is to identify therapeutic agents that increase FMRP from residual *FMR1* messenger (m)RNA, thereby reversing the effects of decreased gene activity. The scope of our project has been to block repressive interactions between microRNAs and the *FMR1* mRNA, thereby increasing protein levels; and to screen a large library (~20,000 compounds) of small molecules for those with the ability to increase FMRP levels. Major findings/accomplishments include: (i) upregulation of FMRP expression by 1.7-fold through the miRNA blocking agents, (ii) utilization of a fast FMRP screen to test 5,000 small molecules for their ability to upregulate FMRP; and (iii) identification of impairments in calcium dynamics, which can be used as a biomarker for treatment approaches. The significance of this work is underscored by the fact that nearly two-thousand children of service personnel are likely to have fragile X syndrome, with a much larger number (~7,500) of active military personnel being carriers of an expanded (premutation) form of the *FMR1* gene.

15. SUBJECT TERMS
Fragile X, autism, *FMR1*, FXTAS, CGG repeat, epilepsy, seizures, FMRP, PTSD, premutation, iPSC, progenitor, calcium regulation

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1. INTRODUCTION

Fragile X syndrome (FXS) is the most common heritable form of intellectual disability, the most common single-gene form of autism, and a relatively common cause of epilepsy. The syndrome is caused by partial or complete silencing of the fragile X (*FMR1*) gene when a CGG-repeat element in the gene expands to more than 200 repeats, leading in turn to loss of the *FMR1* protein (FMRP). The protein is important for brain development, and its loss is accompanied by both intellectual and behavioral disability. Accordingly, the central objective of the proposed research is identification of therapeutic agents that stimulate production of FMRP from residual *FMR1* messenger (m)RNA in neurons, thereby reversing the effects of decreased gene activity. Our approach is twofold: (i) to block repressive interactions between microRNAs and the 3′ non-coding portion of the *FMR1* message, thereby leading to increased protein levels; (ii) to screen a large library (~20,000 compounds) of small molecules, each having the potential for crossing the blood-brain–barrier, for those with the ability to increase FMRP levels. Increasing the expression of FMRP holds the potential to correct ALL of the clinical domains of fragile X syndrome, including epilepsy-like activity observed for both those with FXS and carriers of smaller CGG-repeat expansions. Finally, posttraumatic stress disorder (PTSD) has been described in fragile X syndrome and in premutation carriers. Thus, the proposed studies may lead to treatments that reduce the PTSD risk as well, an issue of importance for military personnel. Since the prevalence of fragile X syndrome is approximately 1 in 3,000 to 4,000 in the general population, nearly two-thousand children of service personnel are likely to have fragile X syndrome, with a much larger number (~7,500) of active military personnel being carriers of an expanded (premutation) form of the *FMR1* gene.

2. KEYWORDS

Fragile X, autism, *FMR1*, FXTAS, CGG repeat, epilepsy, seizures, FMRP, PTSD, premutation, iPSC, progenitor, calcium regulation

3. OVERALL PROJECT SUMMARY

Task 1. Identification of *cis*-elements for translational up-regulation within the *FMR1* mRNA non-coding regions.

Objective: The objective of this task was to capitalize on inhibitory interactions between regulatory elements within the *FMR1* mRNA (5′- and/or 3′ untranslated regions) to increase the efficiency of translation from the native mRNA. For a portion of these studies, we would use a surrogate (reporter) that possessed *FMR1* 5′ and 3′ untranslated regions, which harbor most of the cis-regulatory sequence elements of the mRNA.

Summary of Results/Progress: For studies under this task, we utilized a surrogate (reporter) mRNA that contains all elements of the *FMR1* mRNA sequence except for the substitution of a reporter protein (eGFP) coding region for the FMRP coding region; thus, all regulatory elements of the *FMR1* gene are present. This reporter plasmid was constructed and the construct transfected into control and expanded-CGG-repeat–bearing human fibroblast lines. The transfection efficiency was typically ~50% as determined by *in situ* eGFP when examined under epifluorescence.

Although our transfection protocol was optimized for fibroblasts, this protocol produced significant toxicity when applied to neurons derived from either human iPSCs or the mouse premutation model. We therefore piloted a second method that uses the cell penetrating peptide, penetratin (review: (Dupont et al., 2011)). Four day-in-vitro (DIV) mouse neurons were exposed to penetratin conjugated to Alexa 488 fluorophore for 24 hours. Optimization of the peptide concentration and exposure protocol yielded over 40% of the neurons showing strong internalization of the dye into the cytosol. Our intention was to use penetratin to target *FMR1* mRNA to our neuronal cell models after additional optimization to achieve at least 50% transfection efficiency in the absence of detectable toxicity.

During the course of these experiments, we realized that an important approach to the same problem would be to use the native protein (FMRP) as a “reporter” for blocking of various targets along the 3′UTR of the *FMR1* message. In other words, as a complement to the studies with the exogenous (eGFP) reporter, we could use synthetic RNA analogues (e.g., BNAs; (Prakash, 2011)) to block the target sequences of one or more microRNAs along the *FMR1* 3′UTR. This approach does not suffer the adverse effects (partial transfection, transfection toxicity) associated with the eGFP reporter.

In the first component of these experiments, we determined the profile of all micro(mi)RNAs in the cell types under study; the idea being that it would do no good to block a site for which there was no appreciable miRNA. For the next component, we analyzed (*in silico*) the entire 2.2kb of the 3′UTR to determine which sequence

elements fit criteria for miRNA binding (including strength of binding; ~100 targets). The combination of miRNA concentrations x target binding strength yielded a predicted set of targets for blocking.

On the basis of this information, we designed a set of BNAs to act as target blockers and measured the change of endogenous FMRP following addition of the BNAs, singly or in combination. The best target blocker was one that prevented the binding of miR19b, resulting in an average increase of ~70% in the level of FMRP. However, an unexpected observation was that part of this increase is likely due to protection of the mRNA itself from degradation, presumably through the action of miR19b in an RNAi capacity. These experiments represent a substantial completion of Task 1; however, additional experiments would be warranted to determine to what extent greater coverage of miRNA binding sites (simultaneous binding of multiple target blockers) further increase FMRP levels through a combination of increased mRNA stability and direct translational upregulation.

Summary of Accomplishments/Discussion – Following studies using a variety of oligomers to block the microRNA-mRNA interactions, and using various means for delivery, the best outcome was about 1.8-fold increase in eGFP reporter fluorescence; eGFP was the reporter for the plasmid construct with the *FMR1* 3′UTR, the target of the microRNAs. Although the increase was measurable and significant, our experiments suggested that there would be two important hurdles to the use of blocking oligomers as a treatment strategy. Specifically, the oligos are difficult to deliver to cells, and likely would be impractical as a therapeutic agent, given the modest increase observed in the cell-culture model. This last issue may well be circumvented with the latest-generation modified oligos; however, such molecules are too expensive at present to be supported by the grant. Notwithstanding these caveats, the studies were designed as a proof-of-principle to the upregulation of the *FMR1* gene through inhibition of repressive elements in the 3′UTR of the gene. We have succeeded with this task.

Task 2. Small molecule screening of a specific (CNS-set Chembridge) library of ~20,000 small molecules for candidates capable of increasing FMRP and eGFP levels in transfected fibroblasts.

Objective: As an alternative to the screening strategy under Task 1, the central objective under the current task was to screen a large collection of small chemical compounds with the goal of identifying species capable of inducing FMRP production, without initial regard to mechanism. The main inclusion criterion for the compound library was their increased probability of crossing the blood-brain-barrier. Each drug (in DMSO) was plated into 96-well culture plates in the presence of fibroblasts from a premutation fragile X carrier who is known to produce only 30-40% of the normal level of FMRP.

Summary of Results/Progress: Although we had originally proposed using a library of 50,000 compounds, the smaller number of compounds (20,000) reflected the increased price of each compound for the formulation required for our experiments. Our original approach was to use each drug (in DMSO), plated into 384-well culture plates, followed by the addition of (eGFP transfected) fibroblasts; in the interim, we found a far faster method for detecting endogenous FMRP levels, a FRET-based approach originally described by Schutzius et al. (2013), and modified by Cisbio (Cisbio US, Bedford, MA). The idea behind this approach is that a protein can be detected if fluorescence donor- and acceptor-labeled antibodies are bound to the same protein. This method is very rapid, has extremely low background, and is very sensitive to low levels of protein. The added bonus is that it would eliminate the need to use an eGFP reporter for a first step and a Western blot analysis for a second step – the assay can be performed on native FMRP in a single step, in a single well for each compound. We therefore developed the method in our laboratory prior to screening the compounds.

During the second year of the project, the FRET-based approach was fully implemented and validated, and has allowed us to analyze 4,920 chemical compounds to date. One significant problem with the analysis, not anticipated at the time of writing the original proposal, was that none of the robotic sample handlers available on campus were capable of sterile handling of mammalian cells (this was a surprise to us, since the situation had been represented differently). This meant that the technician working on the project had to perform all liquid-handling procedures by hand.

One huge advantage of the modified approach using FRET and the native fibroblast lines is that it allowed us to use fibroblast lines with expanded CGG repeats (and low FMRP levels); thus, we could assess the potential for increased FMRP expression under conditions where its expression is known to be diminished. The FRET method, and the 384-well format, also allowed us to run multiple replicates of each compound, as well as test the effects of the carrier (dimethyl sulfoxide, DMSO) on FMRP expression.

In the first phase of our screening approach, we spent substantial time developing protocols for ensuring uniform response of the assay, which reflects consistent numbers of cells cultured in each of the 384 wells, and consistent delivery of lysing and detection solutions. Based on our analysis of multiple control samples across plates, we used a value of 2.0 as the cutoff for "hit" compounds and $p < 0.001$ from one-way ANOVA for each plate.

We observed that for most of the ~5,000 test compounds analyzed to date, there was little variation in the relative FRET compared to the DMSO controls. This finding indicates that most compounds have little effect of FMRP expression, either because they intrinsically lack any effect on FMRP expression, or they may not be penetrating the cells in culture. For each plate of 80 compounds, we seeded two 96-well plates at ~7,500 cells per 200 µl RPMI and grew them overnight, followed by serum starvation for 16 hr. The cells were then treated with 20 µM of each compound (or DMSO only) containing 0.05% DMSO in RPMI and were incubated for a further 24 hr. The cells were then lysed, followed by FRET assay analysis.

Using this approach, we identified ~50 "hits" on the primary screen that satisfied both the criterion that the mean elevation exceeds 2.0-fold and that $p < 0.001$, corrected for multiple outcomes through the plate-wise ANOVAs (e.g., **Figure 1**). We later began to run secondary tests on the primary hits as we continued to perform the primary screen. Although the secondary screens initially tended to bear out the initial observations, we noted more recently that the degree of induction – measured by parallel Western blot of the primary hit compounds – did not manifest the same degree of induction. This result, which resulted in some primary hits being eliminated, now appears to be due to interference with the assay because of intrinsic fluorescence of some of the compounds. We do not yet understand this confound, since our choice of the FRET method was based on the elimination of any signal from fluorescent compounds. This issue is currently being investigated.

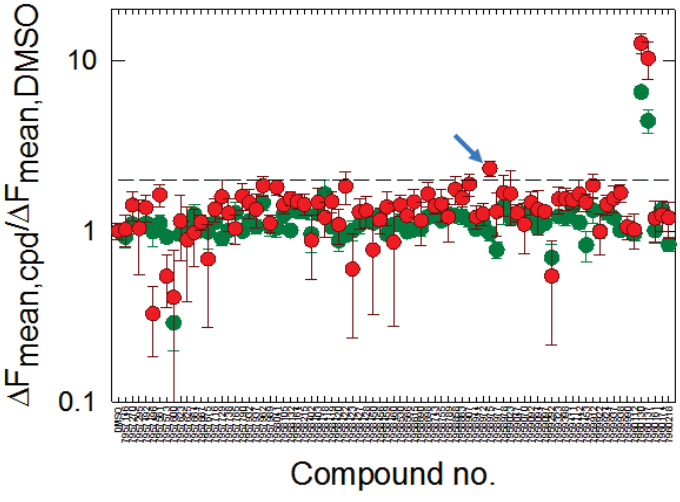

Figure 1. Semi-log plot demonstrating consistency of expression levels upon performing a repeat FRET analysis. Note that nearly all observations fall below a 2-fold increase (dashed line), except for the dramatically elevated level for a single sample (green); a repeat analysis yielded even higher values for that same sample, which is regarded as a "hit." One additional sample (arrow) slightly exceeds the 2-fold threshold for one analysis, but does not meet that threshold for the other analysis.

Summary of Accomplishments/Discussion – We have made two significant accomplishments related to this Task: (i) we have implemented and optimized a FRET-based assay that is now capable of screening our compound library for small molecules that result in an increase in FMRP in a live cell culture; (ii) we have identified a number of hit compounds that are capable of increasing FMRP levels in fibroblasts from an affected individual, where FMRP production is known to be impaired. Before we can comment further on the potential utility of any of the hit compounds, we will need to perform a number of additional studies to gain some idea of the basis for their action(s). Are they influencing transcription? Are they stimulating production of FMRP specifically or are they simply enhancing translation in general? What is their effective range and time-course of effect, and so forth? These questions should be addressed in follow-up investigations.

Task 3. ***Creation of iPSC-derived and transdifferentiated neurons from fibroblast lines***

Objective: The principal objective of this task was to generate neuronal cell models that express *FMR1* CGG-expansion repeats in the high premutation to full-mutation range using two approaches: (1) reprogramming fibroblasts to iPSCs and then promoting neuronal cell differentiation (i.e., establishing neural precursor cell (NPC) lines); and (2) direct trans-differentiation of fibroblasts to a neuronal cell lineage. Using those and other (mouse) neuronal cell models, we proposed to perform transcriptional, morphological, and functional characterization of human iPSC-derived NPCs and transdifferentiated neuronal cells using single cell analyses of transcriptional and protein markers, microscopic fluorescence video imaging of Ca^{2+} dynamics, and action potential properties measured by current clamp. We proposed similar studies of mouse hippocampal neuronal cultures derived from knock-in premutation mouse with >200 CCG-repeat expansion, all

with the goal of examining the antagomiRs (Task 1) and small molecules (Task 2). Begin screening small molecules and antagomiRs in mouse neural networks.

Summary of Results/Progress: As noted above, this task focuses on generating neuronal cell models that express *FMR1* CGG-expansion repeats in the high premutation to full-mutation range. Within the project period, we have subcloned several fibroblast lines from individuals with *FMR1* alleles in the normal range (<35 CGG repeats), the mid-repeat range (40-100 CGG repeats), the high-repeat range (150-200 CGG repeats), and the FXS range (>200 repeats).

We have determined that CGG expansion repeat size and FMRP expression levels remain consistently stable under established culture conditions for each fibroblast clonal line. A new and possibly complicating finding, particularly in the premutation ranges, is that *FMR1* mRNA can vary depending on culture conditions, including time out from media changes, days in culture, and passage number. Since mRNA levels are an important determinant of FMRP levels in human patients, this new observation underscores the importance of maintaining stringent experimental culture protocols when performing *in vitro* studies. Our findings indicate that there are alterations in both basal and maximal mitochondrial respiratory capacity in fibroblasts expressing *FMR1* CGG expansions.

During the first year of the project, we focused on a comparative analysis of two neuronal culture models of *FMR1* trinucleotide expansion disorders. These studies expanded on our initial report identifying morphologic and functional differences between an isogenic pair of iPSC-derived NPCs derived from a mosaic *FMR1* premutation female patient (Liu et al., 2012), postmortem human brain (Pretto et al., 2014), and similar abnormalities in the mouse premutation model (Cao et al., 2012; Kaplan et al., 2012; Cao et al., 2013). A detailed analysis has defined in some detail how *FMR1* CGG-expansion size and developmental age in culture influence levels of *FMR1* mRNA, FMRP, and the patterns of neuronal morphology, including dendritic length and number per neuron (**Figure 2**).

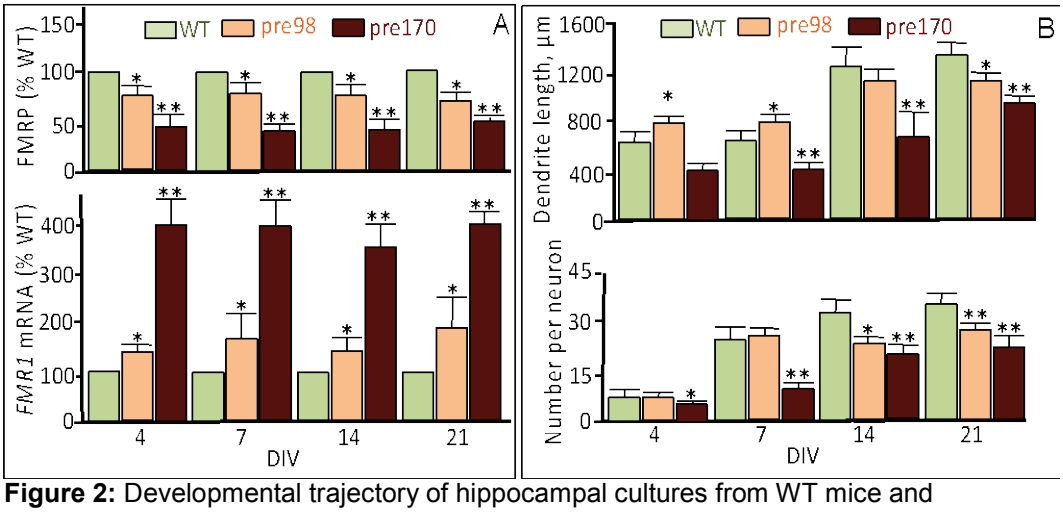

Figure 2: Developmental trajectory of hippocampal cultures from WT mice and premutation KI mice expressing mid (pre98) and high (pre170) CGG repeats. (A) FMRP and *FMR1* mRNA levels relative to WT. (B) Total dendritic length and number of branches (termini) per neuron. Mean ±SEM **p*<0.05; ***p*<0.01.

Neuronal *FMR1* mRNA levels are highest in pre170 (3-4 fold of WT) and intermediate in pre98 (1.5-2 fold of WT) neurons, whereas FMRP levels are lowest in pre170 (~50% of WT) and intermediate in pre98 (75–90% of WT). These key genotype differences are seen as early as 4 DIV and extend throughout the critical timeframe needed for neuronal cultures to develop sufficient dendritic complexity (**Figure 2**) and synaptic architecture (Chen et al., 2010) to engage spontaneous synchronous Ca^{2+} oscillations (SCOs; **Figure 3**) and electrical field potentials (Cao et al., 2012) across the networks they form. Our data indicate that the onset and progression of both morphological and functional abnormalities is highly dependent on the size of the *FMR1* CGG expansion, with pre98 neurons (**Figure 2, B**, upper panel) initially extending longer dendrites with similar number of dendritic branches (**Figure 2, B**, lower panel) compared to WT at 4 and 7 DIV. By 21 DIV, both dendritic length and number of branches are significantly less than WT, which is sharply contrasted by the pre170 neurons that produce less developed dendritic processes than either pre98 or WT neurons throughout the developmental timeframe measured to date (**Figure 2, B**). Such genotype-dependent differences in development of neuronal complexity are not limited to *in vitro* cell cultures, as they have been detected in Golgi stains of premutation mouse brain sections (Wenzel et al., 2010; Berman et al., 2012), and altered neuroplasticity in acute hippocampal slice preparations (Hunsaker et al., 2012).

We directly compared the patterns of SCOs and responses to glutamate challenges elicited by hippocampal neurons cultured from the premutation mouse with iPSC-derived NPCs at critical periods of neurodevelopment. Two observations consistent between the murine and human *in vitro* models significantly differ between *FMR1* premutation and their corresponding normal neurons: (1) significantly higher frequency and clustering of Ca^{2+} oscillations in premutation neurons, and (2) significantly prolonged responses to challenges with glutamate.

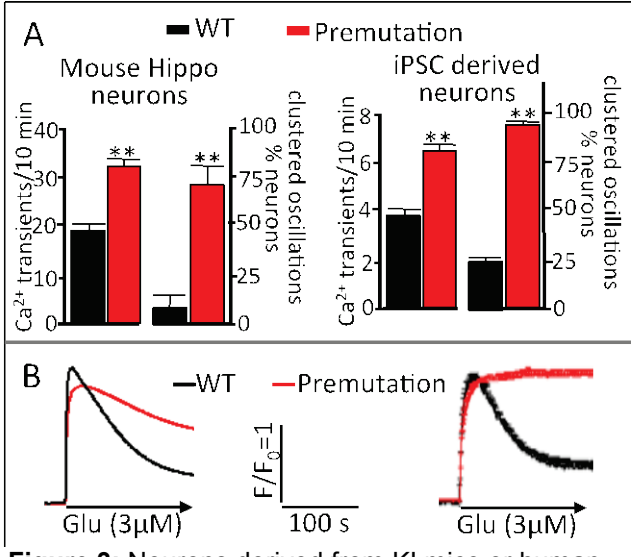

Figure 3: Neurons derived from KI mice or human iPSCs exhibit early (7DIV) abnormal Ca^{2+} dynamics (A) and response to Glu (B).

We also obtained evidence that abnormal glutamate transport is common both in human (Pretto et al., 2014) and mouse (Cao et al., 2013) *FMR1*-premutation lines. The use of Ca^{2+}-selective microelectrodes to record resting free cytoplasmic Ca^{2+} in the presence of TTX (to block SCOs) indicated that premutation neurons cultured from mice maintain chronically elevated cytoplasmic free Ca^{2+} compared to wild type neurons. Such alterations in Ca^{2+} dynamics would be expected to not only influence activity dependent dendritic growth patterns, but also influence Ca^{2+}-dependent signaling essential for maintaining normal synaptic connectivity.

To further assess the potential impacts of altered Ca^{2+} dynamics observed with premutation neurons, we investigated whether the levels and patterns of expression of FMRP and *FMR1* mRNA influence those of Cdk5 and ATM, two transcriptional factors critical for orchestrating nuclear and synaptic signals that maintain influence long-term neuronal connectivity and neuronal survival, that when disturbed, may promote progressive neuronal damage in a feed-forward manner. Consistent with Western blot data, immunofluorescence labeling of cultured hippocampal neurons from premutation mice showed ~40–50% reduction of FMRP within soma. However, the levels of FMRP within the distal dendrites of premutation neurons are less than 15% than that found in wild-type neurons (**Figure 4).**

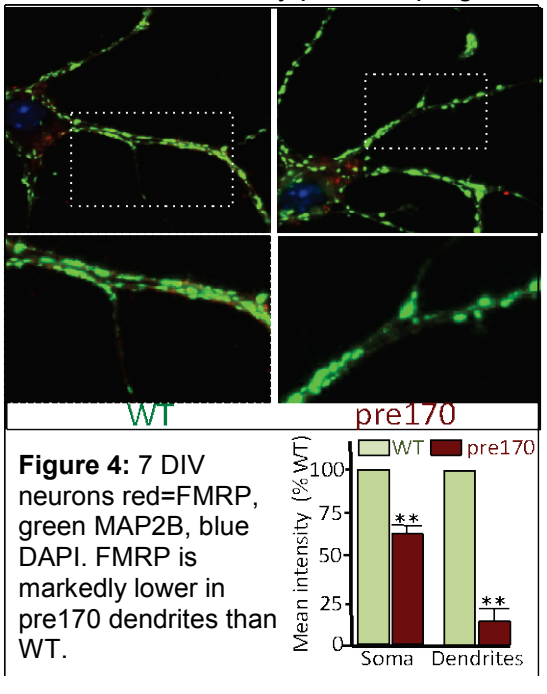

Figure 4: 7 DIV neurons red=FMRP, green MAP2B, blue DAPI. FMRP is markedly lower in pre170 dendrites than WT.

A potentially significant finding linking chronic Ca^{2+} dysregulation, abnormal synaptic connectivity (i.e., altered responses to Glu) and progressive impairments in neuronal health, is that high premutation neurons (CGG >170) express much higher levels of both Cdk5 (~2-fold of wild type) and ATM. Importantly, initial results indicate that phospho-ATM is upregulated ~5.5-fold in high premutation cells compared to wild type (**Table 1**).

Taken together, these are the first results to demonstrate a molecular signaling mechanism that involves chronic activation of two transcriptional factors concomitant with an abnormal Ca^{2+} dynamics and distribution of FMRP in high-range premutation neurons. Upregulation and activation of Cdk5 and ATM, possibly the consequence of chronically elevated neuronal Ca^{2+}, can produce feed-forward signaling that alters not only the integrity of nuclear DNA repair but also alteration in synaptic connectivity.

We have more recently (unpublished) identified mechanistically linked biomarkers that are early etiological drivers of CGG-repeat-expansion–related neurological developmental impairments. The earliest of these is chronically elevated cytoplasmic Ca^{2+} and μ-calpain activation. We hypothesized that chronically activated μ-calpain would have a profound influence on the level of activated Cdk5 due to a prolonged shift in the balance of p35/p25, whose ratio tightly regulates the activity of Cdk5 during prenatal and postnatal development of the brain. Significantly, the activity of this multifunctional signaling kinase (Cdk5) is critical not only for proper synaptogenesis and neural network formation, but its unregulated activity is known to promote abnormal signaling to the nucleus and alter ATM phosphorylation that can influence temporal transcription patterns of

key synaptic proteins. We do not know the relationship between FMRP loss and these signaling abnormalities; however, they provide us with a range of outcome measures to test the effects of increasing FMRP.

Under the current Task, we continued our investigation of key Ca^{2+}-regulated pathways leading to abnormal activation of μ-calpain–CdK5–ATM signaling to the nucleus, which leads to mitochondrial oxidative stress and cellular Ca^{2+} overload. Here we obtained new mechanistic data implicating this pathway in not only early impairments in morphological development, but also impaired glutamatergic neurotransmission. We discovered consistent patterns of developmental dysfunction across the mouse *FMR1* KI mouse model and iPSC-derived neuronal precursor cells (NPCs) from *Fmr1* patients with either premutation or full-mutation alleles.

We observed that intracellular cytoplasmic Ca^{2+} is chronically elevated as early as 7 days in vitro and that these Ca^{2+} levels appeared to correlate with the level of FMRP deficiency, with human full-mutation cells having the highest levels and greatest FMRP reduction. Importantly, elevated cytoplasmic Ca^{2+} and μ-calpain activity were coincident with reductions in the ratio of P35/P25, which relieves the inhibition of Cdk5. One major substrate of Cdk5 is nuclear ATM, and we observed that, as predicted, cortical tissues from brains of mice deficient in expression of FMRP (preCGG mice) have significantly higher levels of phosphorylated ATM (p-ATM) compared with wild-type mice at the same age.

Upon closer examination of the distribution of ATM within neurons using confocal microscopy, we discovered a pattern of intranuclear puncta that are brighter and more abundant in FMRP-deficient neurons compared to wild-type neurons (**Figure 5**).

Finally, we discovered evidence for abnormal glutamatergic neurotransmission that extends across mouse and human models, providing a functional biomarker associated with FMRP deficiency, a biomarker that lends itself to rapid throughput screening of small molecules that normalize FMRP levels (Task 2).

***Summary of Accomplishments/Discussion*:** Collectively, concordant results from both our mouse and human models of FMRP deficiency provide an unprecedented opportunity to screen small molecule leads (increase in FMRP) identified under Task 2. We have identified morphological, biochemical, and functional biomarkers that not only are likely to make etiological contributions to the developmental impairments produced by FMRP deficiency, but also will facilitate targeted screening of small molecules that could ameliorate the onset or severity of these biomarkers.

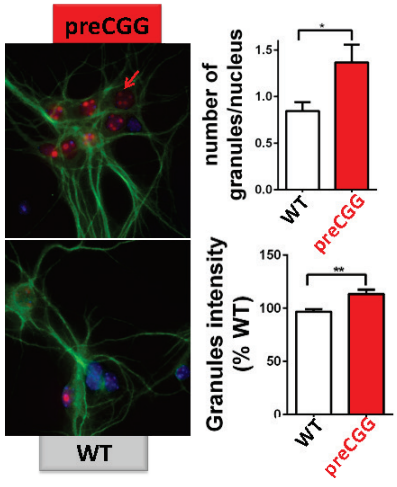

Figure 5. ATM puncta (red) in a premutation CGG-repeat (preCGG) allele, compared to a wild-type (WT) allele, using confocal microscopy in mouse neurons.

Collectively, our experimental models suggest that they would be useful in defining common etiological mechanisms that promote early-onset developmental impairments in neuronal connectivity and later progression of neuropathological sequelae. Importantly, our mouse and human models underscore the translational potential of screening small molecule libraries that enhance expression of FMRP (described under Task 2), during the entire prenatal and postnatal developmental timeframe, for their therapeutic potential in normalizing morphometric, signaling, and synaptic functions in an iterative manner. Unfortunately, given the time involved with the primary and secondary screening of the chemical compounds, we did not have time to mount a systematic study of the effects of the compounds on mouse phenotype.

Task 4. *Extension of small molecule targeting of the FMR1 mRNA in premutation (preCGG) mouse and human iPSC and transdifferentiated neurons*.

Objective: The primary objective of the entire project is the identification and further characterization of small molecules that are capable of increasing FMRP levels in neuronal cells (mouse or human iPSC-derived cells). The largely negative results of Task 1 (modest 1.8-fold increase in FMRP), coupled with the anticipated difficulties of delivery of oligomeric RNA molecules to the CNS, suggest that further effort under Task 1 would not be fruitful. Moreover, the exciting results under Task 2, with a number of compounds (all with predicted ability to cross the blood-brain-barrier) identified that are capable of increasing FMRP levels, have positioned us to study the effects of those compounds *in vivo*. The development of highly sensitive outcome measures under Task 3 would allow us to proceed to Task 4, focused entirely on the “hit compounds” of Task 2. Thus,

the principal objective under Task 4, while largely unchanged from the original proposal, was shifted to focus on candidate small molecules.

***Summary of Results/Progress*:** As noted above, since we only recently identified suitable "hit" compounds for further study, and are still in the process of trying to separate non-FRET contributions to fluorescence from some of the "hit" compounds, we have not been able to substantively tackle Task 4.

Summary of Accomplishments/Discussion: Nothing to report.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Quantified the effects of interfering with the miRNA-3′UTR interactions for the *FMR1* gene; the best target blocker was one that resulted in an average increase of ~70% in the level of FMRP. An unexpected observation was that part of this increase is likely due to protection of the mRNA itself from degradation.
- Developed and implemented a much faster method for quantifying the effects of various agents on the level of FMRP using a FRET-based assay that allows rapid quantification of endogenous FMRP.
- Completed subcloning of human fibroblasts from normal and *FMR1* premutation individuals with *FMR1* expansion repeats in the mid-, high-, and full range.
- Successfully obtained iPSCs and a pair of isogenic premutation iPSC-derived NPCs from a mosaic premutation patient.
- Identified that similar functional impairments in Ca^{2+} dynamics occur in both mouse premutation neurons and human iPSC-derived NPCs.
- Identified evidence for mitochondrial dysfunction in mouse premutation neurons.
- Discovered that although FMRP is modestly (25–40%) reduced relative to wild type, mouse high-range premutation neurons have nearly 7-fold less FMRP in their distal dendrites.
- Preliminary results indicate that high-range premutation neurons in culture (and brain tissue) have increased levels of Cdk5, ATM, and P-ATM compared with respective preparations from wild type.
- We have identified a number of small molecules that, upon single dose of the compound to patient fibroblasts in culture, have raised the level of FMRP by more than 2-fold within a 24-hr period.
- Under Task 3, we have developed a series of refined outcome measures in neuronal cells (mouse and human iPSC-derived neurons) to allow us to evaluate the potential therapeutic benefit of "hit" compounds identified under Task 2.

5. CONCLUSION

Our discovery of a small number of "hit" compounds under Task 2 is potentially extremely important for targeted treatment for fragile X syndrome, the leading single-gene form of intellectual disability and leading known form of autism. The "potential" nature of these findings reflects the need for follow-on studies to characterize the mechanism of action of the chemical compounds, their selectivity (increase only FMRP, or other proteins as well?), evidence of toxicity (we have not seen any evidence of reduced cell viability) in both cell and animal models, evidence for distribution to the brain, and finally, evidence of FMRP up-regulation in the animal model.

Fundamentally, we now have the potential for the development of a targeted therapeutic approach to the most common single-gene disorder giving rise to cognitive impairment and autism. Beyond this, elevation of FMRP would hold the potential for the treatment of both PTSD and one or more of the major psychiatric disorders, based on the observations (for the latter) that FMRP levels are decreased in the brains of individuals who suffered from major depression, bipolar disorder, and schizophrenia.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

a. *Manuscripts submitted for publication during the period covered by this report resulting from this project.*

1. Lay Press: N/A

2. Peer-Reviewed Scientific Journals:

Berman RF, Buijsen RA, Usdin K, Pintado E, Kooy F, Pretto D, Pessah IN, Nelson DL, Zalewski Z, Charlet-Bergeurand N, Willemsen R and Hukema RK (2014) Mouse models of the fragile X premutation and fragile X-associated tremor/ataxia syndrome. *J Neurodev Disord* 6:25. PMID25136376, PMC4135345

3. ***Invited Articles:*** N/A
4. ***Abstracts:*** N/A

b. ***Presentations made during the last year (international, national, local societies, military meetings, etc.).***

Gaëlle Robin, Jose R. López, Susan Hulsizer, Paul J. Hagerman, Isaac N. Pessah Ataxia-telangiectasia mutated (ATM) signaling is up-regulated in an *FMR1* premutation mouse model. Keystone Symposium - Pathways of Neurodevelopmental Disorders, March 17, 2015 Tahoe City, California, USA.

7. INVENTIONS, PATENTS AND LICENSES

Nothing to report

8. REPORTABLE OUTCOMES

Reportable outcomes are in process for publication and completion of experiments (post-project) that will allow publication.

9. OTHER ACHIEVEMENTS

Nothing to report

10. REFERENCES

Berman RF, Murray KD, Arque G, Hunsaker MR and Wenzel HJ (2012) Abnormal dendrite and spine morphology in primary visual cortex in the CGG knock-in mouse model of the fragile X premutation. *Epilepsia* 53 Suppl 1:150-160. http://www.ncbi.nlm.nih.gov/pubmed/22612820

Cao Z, Hulsizer S, Cui Y, Pretto DL, Kim KH, Hagerman PJ, Tassone F and Pessah IN (2013) Enhanced asynchronous Ca^{2+} oscillations associated with impaired glutamate transport in cortical astrocytes expressing *FMR1* gene premutation expansion. *J Biol Chem* 288:13831-13841. PMCID: 3650419

Cao Z, Hulsizer S, Tassone F, Tang HT, Hagerman RJ, Rogawski MA, Hagerman PJ and Pessah IN (2012) Clustered burst firing in *FMR1* premutation hippocampal neurons: amelioration with allopregnanolone. *Hum Mol Genet* 21:2923-2935. PMCID: 3373240

Chen Y, Tassone F, Berman RF, Hagerman PJ, Hagerman RJ, Willemsen R and Pessah IN (2010) Murine hippocampal neurons expressing *Fmr1* gene premutations show early developmental deficits and late degeneration. *Hum Mol Genet* 19:196-208. PMCID: 2792156

Dupont E, Prochiantz A and Joliot A (2011) Penetratin story: an overview. *Methods Mol Biol* 683:21-29. http://www.ncbi.nlm.nih.gov/pubmed/21053119

Hunsaker MR, Kim K, Willemsen R and Berman RF (2012) CGG trinucleotide repeat length modulates neural plasticity and spatiotemporal processing in a mouse model of the fragile X premutation. *Hippocampus* 22:2260-2275. PMCID: 3449027

Kaplan ES, Cao Z, Hulsizer S, Tassone F, Berman RF, Hagerman PJ and Pessah IN (2012) Early mitochondrial abnormalities in hippocampal neurons cultured from *Fmr1* pre-mutation mouse model. *J Neurochem* 123:613-621. PMCID: 3564636

Liu J, Koscielska KA, Cao Z, Hulsizer S, Grace N, Mitchell G, Nacey C, Githinji J, McGee J, Garcia-Arocena D, Hagerman RJ, Nolta J, Pessah IN and Hagerman PJ (2012) Signaling defects in iPSC-derived fragile X premutation neurons. *Hum Mol Genet* 21:3795-3805. PMCID: 3412379

Prakash TP (2011) An overview of sugar-modified oligonucleotides for antisense therapeutics. *Chem Biodivers* 8:1616-1641. http://www.ncbi.nlm.nih.gov/pubmed/21922654

Pretto DI, Kumar M, Cao Z, Cunningham CL, Durbin-Johnson B, Qi L, Berman R, Noctor SC, Hagerman RJ, Pessah IN and Tassone F (2014) Reduced excitatory amino acid transporter 1 and metabotropic glutamate receptor 5 expression in the cerebellum of fragile X mental retardation gene 1 premutation carriers with fragile X-associated tremor/ataxia syndrome. *Neurobiol Aging* 35:1189-1197. PMCID: 4062976

Schutzius G, Bleckmann D, Kapps-Fouthier S, di Giorgio F, Gerhartz B and Weiss A (2013) A quantitative homogeneous assay for fragile X mental retardation 1 protein. *J Neurodev Disord* 5:8. PMCID: 3635944

Wenzel HJ, Hunsaker MR, Greco CM, Willemsen R and Berman RF (2010) Ubiquitin-positive intranuclear inclusions in neuronal and glial cells in a mouse model of the fragile X premutation. *Brain Res* 1318:155-166. PMCID: 3086812

11. **APPENDICES**

Nothing added

What individuals have worked on the project?

Name:	Paul Hagerman
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1.2
Contribution to Project:	Dr. Paul Hagerman and his laboratory performed all analyses of FMRP, directed the miRNA blocking experiments, and ran all of the small molecule screening experiments.
Funding Support:	NIH, UC Davis
Name:	Isaac Pessah
Project Role:	Co-PI
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	2.4
Contribution to Project:	Dr. Pessah and his laboratory performed all experiments with tissue culture, including neural precursor cells and primary neuronal culture with the knock-in mice as well as inducible high CGG mRNA mice. His laboratory also performed westerns blot analysis and immunohistochemistry assays on these mouse lines.
Funding Support:	NIH, EPA
Name:	Randi Hagerman
Project Role:	Investigator
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	0.36
Contribution to Project:	Dr. Randi Hagerman was responsible for providing the proper clinical oversight and for continuing to provide the cell resources and clinical phenotypes for the skin biopsy-derived fibroblasts used in the current study.
Funding Support:	NIH, HRSA, Pharmaceutical companies (Neuren, Curemark, Novartis, Hoffman-LaRoche)
Name:	Glenda Espinal
Project Role:	Staff research assistant
Researcher Identifier (e.g.	NA

ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Principal research associate who performed all of the FMRP-FRET screening operations under Dr. P. Hagerman's supervision.
Funding Support:	NIH (sometimes less than 12 mos effort)
Name:	Anna Ludwig
Project Role:	Graduate student/postdoc
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	12
Contribution to Project:	Dr. Anna Ludwig was a graduate student, then postdoc who participated in the early studies of miRNA inhibition.
Funding Support:	NA
Name:	Edwin Chuck
Project Role:	Graduate student
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	6
Contribution to Project:	A graduate student who continued the studies of miRNA inhibition.
Funding Support:	NA
Name:	Yi Mu
Project Role:	Staff statistician
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1
Contribution to Project:	Staff statistician from Division of Biostatistics who provided all of the expertise in proper statistical analysis.
Funding Support:	NIH
Name:	Dalyir Pretto
Project Role:	Postdoctoral scholar
Researcher Identifier (e.g. ORCID ID):	NA

Nearest person month worked:	1
Contribution to Project:	Worked with Glenda Espinal to screen the compounds (grow up cells, treat, assay with Cisbio FRET assay) with FRET assay to measure FMRP levels.
Funding Support:	NIH
Name:	Susan Hulsizer
Project Role:	Project scientist
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	3
Contribution to Project:	General culture support and lab management on the project in the Pessah Lab
Funding Support:	NIH, UC Davis
Name:	Gaelle Robin
Project Role:	Post-Doctoral Scholar
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	9
Contribution to Project:	Contributed to research performed in furtherance to Task 3.
Funding Support:	NIH
Name:	Gennady Cherednychenko
Project Role:	Project Scientist
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	5
Contribution to Project:	General imaging support
Funding Support:	EPA, NIH
Name:	Shane Antrobus
Project Role:	Staff research assistant
Researcher Identifier (e.g. ORCID ID):	NA

Nearest person month worked:	2
Contribution to Project:	Helped with genotyping the mice
Funding Support:	NIH
Name:	Yao Dong
Project Role:	Staff research assistant
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1
Contribution to Project:	General tech support in Pessah Lab for the research performed under Task 3 in Pessah lab
Funding Support:	NIH, EPA
Name:	Kenneth Todd
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1
Contribution to Project:	Helped with imaging for Task 3 in Pessah lab
Funding Support:	NIH
Name:	Rui Zhang
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1
Contribution to Project:	Helped with live Calcium imaging under Task 3
Funding Support:	NIH, EPA, UC Davis
Name:	Wayne Feng
Project Role:	Project Scientist
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person	1

month worked:	
Contribution to Project:	General electrophysiology support.
Funding Support:	NIH, EPA, UC Davis
Name:	Jeanatan Hall
Project Role:	Undergraduate
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1
Contribution to Project:	Animal care for Task 3
Funding Support:	NIH